The Antiproliferative Effect of Bevacizumab on Human Tenon Fibroblasts Is Not Mediated by Vascular Endothelial Growth Factor Inhibition

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METHODS. Human tenon fibroblast primary cultures were obtained from strabismus surgery subjects. Under low (0.2%) and high (10%) serum conditions, cells were incubated with BVC, ranibizumab (RNB), aflibercept (AFB), or rituximab (RTX) at different concentrations. Total number of cells and number of dead or proliferating (5-bromo-2-deoxy-uridine-positive) cells were assessed after 24 hours. Concentrations of VEGF-A in cell culture media was measured with ELISA. Intracellular IgG was detected with immunostaining and Western blot analysis.

RESULTS. In quiescent hTF culture (0.2% serum) the addition of 5 mg/mL BVC induced widespread cell death. Under proliferative conditions (10% serum), BVC reduced the number of proliferating cells. No such effect was observed with 2.5 mg/mL RNB, although they were equally effective in binding free VEGF-A in the culture media. Instead, the CD20 antibody RTX, which did not bind VEGF, induced hTF death and inhibited proliferation in a BVC-comparable fashion. Bevacizumab, AFB, and RTX were detected intracellularly in a concentration-dependent manner.

CONCLUSIONS. The cell death–inducing and antiproliferative effect of 5 mg/mL BVC appeared not to depend on VEGF inhibition. Our data question a direct role of VEGF for hTF survival and proliferation.

Keywords: human tenon fibroblasts, vascular endothelial growth factor, bevacizumab, glaucoma filtration surgery
known to be associated with bleb failure.\textsuperscript{3,11,16} However, the intracellular pathways affected by anti-VEGF agents in hTFs are largely unknown. The relatively high concentrations of BVC needed to obtain a significant effect, as well as the increased LDH levels in a cell culture experiment\textsuperscript{10} and reports on tissue necrosis after local application of BVC,\textsuperscript{5,17} led us to revisit the mode of action of BVC on hTFs. We hypothesized that BVC does not act through VEGF inhibition but rather via non-antigen-dependent ways.

We investigated the impact of BVC, along with AFB, RNB, and the CD20 antibody rituximab (RTX), on hTFs to distinguish the presumed anti-VEGF effect from possible unspecific modes of action.

**Methods**

**Cell Culture**

Primary cell culture of hTFs was set up from tenon tissue samples of patients who underwent strabismus surgery. Tissue from five patients (two male, three female Caucasians) between 24 and 61 years of age (mean 37 years) was used. All patients had no history of prior ocular surgery or glaucoma. Institutional review board/ethics committee approval for this project, as well as informed consent from each patient, was obtained before conduction of the study (University Medical Center Goettingen; permit no. 8/12/13). The described research adhered to the tenets of the Declaration of Helsinki. Cells were cultivated at 37°C with 5% CO\textsubscript{2} in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% fetal calf serum (FCS), 125 mL/L L-glutamine, and 2.5 mL/L penicillin/streptomycin (all from Aldrich Corp., Munich, Germany). Only cells between cell culture passages 4 to 9 were used. For immunostainings, cells were grown in 24-well plates on 12-mm glass coverslips coated with collagen (collagen solution from bovine skin; Sigma-Aldrich Corp., Munich, Germany) or for Western blot analyses in collagen-coated 35-mm petri dishes. Medium exchange was performed 3 hours after cells were plated with 10 × 4 cells/well. For VEGF inhibition cells were incubated with DMEM (0.2% FCS) containing 2.5, 5, or 10 mg/mL BVC (Roche, Basel, Switzerland); or 5 or 10 mg/mL AFB (Bayer, Leverkusen, Germany); 2.5 mg/mL RNB (Novartis, Basel, Switzerland); or 1, 2.5, or 5 mg/mL RTX (Roche) for 24 hours. For control experiments, equal volumes of PBS were added to the medium. For stimulation, recombinant TGF-β1 (Tebu, Offenbach, Germany) was used at 2 ng/mL final concentration. Purity of the fibroblast cell culture was tested with immunostaining for vimentin. Apoptosis was induced with 1 μM staurosporine (Sigma-Aldrich Corp.).

**Western Blot**

Cells were lysed with radio-immunoprecipitation assay buffer (Sigma-Aldrich Corp.) containing 1% phosphatase inhibitor (Phospho-Stop; Roche) and 1% proteinase inhibitor cocktail (Sigma-Aldrich Corp.). Proteins were separated with 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked at room temperature for 1 hour in 5% BSA (Sigma-Aldrich Corp.) in Tris-buffered saline/0.1% Tween-20 (TBST; AppliChem, Darmstadt, Germany) followed by incubation with the primary antibody in blocking buffer for 1 hour at RT (mouse anti-human IgG, 1:500 [Sigma-Aldrich Corp.]; mouse anti-GAPDH, 1:20,000 [Dako, Glostrup, Denmark]; mouse anti-human CD20, 1:1000 [DAKO, Glostrup, Denmark]). The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Thermo Scientific, Waltham, MA, USA) diluted 1:1000 in TBST and incubated at RT for 30 minutes. Chemiluminescence was obtained with Western blotting detection reagent (ECL-Prime; GE Healthcare, Little Chalfont, UK) and detected with a V3 Western workflow system (Bio-Rad Laboratories, Hercules, CA, USA). All Western blots were performed in duplicate for each sample. Band intensity quantification was performed with ImageJ software (http://image.nih.gov/ij; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry**

Cells were fixed with 2% paraformaldehyde in PBS for 20 minutes, followed by permeabilization with 0.2% Triton X-100 (QBioGene, Carlsbad, CA, USA) in PBS for 5 minutes. The blocking buffer was 1% BSA in PBS, applied for 15 minutes. Primary antibodies were diluted in blocking buffer and incubated for 1 hour at RT (Alexa Fluor 568-conjugated goat anti-human IgG [H+L; Life Technologies, Carlsbad, CA, USA]; monoclonal mouse anti-human CD20cy, 1:50 [DAKO]; mouse anti-vimentin, 1:500 [Abcam, Cambridge, UK]). Secondary antibody was Alexa Fluor 488-conjugated goat anti-mouse IgG 1:1000 (Sigma-Aldrich Corp.). Coverslips were mounted with medium (Mowiol; Roth, Karlsruhe, Germany) containing 5 μL/mL DAPI for nuclei staining.

Staining for dead or viable cells was performed with the live/dead cell imaging kit (Life Technologies) following the kit protocol. Staining for detection of cells in proliferation was performed with 5-Bromo-2-deoxy-uridine (BrDU) Labeling and Detection Kit 1 (Roche).

Cell counts were obtained by manual counting of all fully attached cells exhibiting an intact cytoskeletal pattern in vimentin immunostaining.

**Membrane Filtration of Antibody Solutions**

To separate the antibody from its original buffer, sample volumes of 500 μL pure antibody solution were filtrated through a PVDF membrane centrifugal filter system with a molecular weight cutoff of 30 kDa (Amicon; Merck Millipore) for 10 minutes at 10,000 g at RT. The approximately 370-μL residual antibody solution was resuspended in PBS, and the filtration step was repeated two times. To collect all residual antibody solution the filter was centrifuged in reverse for 2 minutes at 10,000 g, and the antibody solution was diluted back to its initial concentration by filling the volume up to the original 500 μL with PBS. Antibody concentrations were measured with a spectrophotometer (260/280 ratio; Eppendorf, Hamburg, Germany) to ensure equal protein concentration in the original and the buffer-exchanged solutions.

**Vascular Endothelial Growth Factor ELISA**

Vascular endothelial growth factor concentrations in cell culture media were measured using a kit (Quantikine ELISA Kit; R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. All measurements were repeated three times with samples derived from independent experiments.

**Statistical Testing**

Statistical analyses were performed with Prism software, Version 6.0 (GraphPad, La Jolla, CA, USA). All data were presented as mean ± standard error of the mean if not indicated differently.
RESULTS
Concentrations of 5 mg/mL BVC or Higher Led to Rapid Cell Death of Quiescent hTFs
In quiescent hTF cell culture under low-serum condition (0.2% FCS), the addition of 5 mg/mL BVC destroyed the majority of cells within 24 hours. The cells rapidly swelled and eventually disintegrated, resembling necrotic rather than apoptotic cell death, as compared with staurosporine-treated cells (Fig. 1A). Cell counts were highly significantly reduced (1.2 ± 0.8 [BVC] versus 37.1 ± 5.6 cells/mm² [control]; P < 0.0001; ANOVA with Sidak’s multiple comparisons test as used for all subsequent analyses), and the number of dead cells was significantly higher (14.8 ± 1.1 [BVC] versus 3.7 ± 1.2 cells/mm² [control]; P < 0.001; ANOVA). Bevacizumab concentrations of 2.5 mg/mL or lower had no significant influence on the cell number or the number of dead cells (cell count: 37.0 ± 2.6 [2.5 mg/mL BVC] versus 37.1 ± 5.6 cells/mm² [control]; not significant; ANOVA; cell death: 7.1 ± 1.2 [BVC] versus 3.7 ± 1.2; not significant; ANOVA) (Figs. 1B, 1C).

Providing high-serum conditions (10% FCS), 5 mg/mL BVC still reduced the number of cells, but the effect was significantly smaller compared to 0.2% serum (cell counts: 28.2 ± 2.5 [10% + BVC] versus 1.2 ± 0.8 [0.2% + BVC]; P < 0.0001; ANOVA; cell death: 14.8 ± 1.1 [0.2% serum] versus 10.5 ± 0.8 [10% serum]; P < 0.05; ANOVA). The addition of TGF-β1 to 10% media annulled the effect of 5 mg/mL BVC (10% serum + TGF-β1: 37.8 ± 3.4 [BVC] versus 41.8 ± 3.1 cells/mm² [control]; not significant; ANOVA). But a significant reduction in cell number was gained when increasing the BVC concentration to 10 mg/mL (10% serum plus TGF-β1: 27.9 ± 3.0 [BVC] versus 41.8 ± 3.1 cells/mm² [control]; P < 0.01; ANOVA) (Figs. 1B, 1C).

Bevacizumab-Equivalent Concentrations of AFB and RNB Did Not Induce hTF Loss
Assuming that the observed effect was due to VEGF inhibition, we tested whether comparable concentrations of AFB and RNB would lead to similar rates of cell number reduction and cell death increase in low-serum (0.2% FCS) hTF culture. As a first approximation of a comparable VEGF-inhibitory effect, we chose the same volume of antibody solution as used for the 5 mg/mL BVC experiment. Neither 10 mg/mL AFB nor 2.5 mg/mL RNB lead to a significant reduction in cell number (32.4 ± 4.1 [10 mg/mL AFB] versus 32.4 ± 2.3 cells/mm² [control]; not significant; 35.4 ± 2.8 [2.5 mg/mL RNB] versus 32.4 ± 2.3 cells/mm² [control]; not significant) or an increase in the number of dead cells (4.7 ± 1.3 [10 mg/mL AFB] versus 4.5 ± 0.7 cells/mm² [control]; not significant; 4.6 ± 0.8 [2.5 mg/mL RNB] versus 4.5 ± 0.7 cells/mm² [control]; not significant) (Figs. 2A, 2B). Compared to control cells, AFB induced a mild and mostly transient change in cell morphology, with increasing volume of the cell body and thinning of the extensions (Fig. 1A). The morphology of RNB-treated cells was indistinguishable from control cells (Supplementary Fig. S1).

The Detrimental Effect of BVC Is Independent From VEGF Inhibition in the Culture Media
To distinguish VEGF-inhibitory effects from other, unspecific modes of action we measured the amount of VEGF-165 in the cell culture media under various conditions. The VEGF concentration in fresh DMEM cell culture containing 0.2% or 10% serum was not significantly different from zero (2.5 ± 1.3 [P = 0.2] and 1.0 ± 0.3 pg/mL (P = 0.07), respectively; one sample t test; Fig. 3A). Thus, the addition of serum did not add exogenous VEGF to the cell culture.

Instead, the cultured hTFs secreted VEGF, which gradually accumulated in the culture medium (Fig. 3A). After 24 hours, the VEGF concentration in the medium containing low serum was 1.5 ± 1.7 pg/mL (0.2% serum was used as control), and in the high-serum medium was 7.1 ± 3.3 pg/mL (P = 0.002, one sample t test). The addition of BVC at 2.5 and 5.0 mg/mL concentration reduced the levels of free VEGF to almost zero for both low- and high-serum conditions. The same was observed for AFB at concentrations of 5 and 10 mg/mL (Fig. 3B). The higher concentration of BVC and AFB did not further reduce the amount of free VEGF compared to the lower concentration of the antibody (ANOVA with Sidak’s multiple comparisons test: no significant difference between the pairs).

Both antibodies were equally effective in VEGF inhibition, but only 5 mg/mL BVC promoted widespread cell death or reduction of cell number under the low- and high-serum conditions (Fig. 1B). Consequently, the observed effect of 5 mg/mL BVC cannot be attributed to VEGF inhibition. For further investigation of the BVC mode of action, we considered three hypothetical mechanisms: (1) The solvent buffer of BVC is toxic at high concentrations; (2) the effect is IgG-related and not specific for BVC; and (3) the intracellular uptake of BVC causes cell death.

Influence of BVC Solvent Buffer on hTF Culture. The solvent buffer of BVC is acidic (pH = 5.9; data provided from manufacturer). To study whether the observed effect of BVC may be caused by the solvent buffer alone, we extracted all antibody from the BVC solution by filtration through a 30-kD cutoff PVDF membrane. No protein was detected in the filtered buffer by Coomassie staining of gels after SDS-PAGE (data not shown). The pure solvent buffer was then added to the cell culture in a volume equivalent to 5 and 10 mg/mL BVC. Compared to control cell culture where the same volume of PBS was added, the BVC solvent buffer (5 mg/mL equivalent) significantly increased the number of dead cells (Fig. 4B) after 24 hours of incubation (4.6 ± 0.9 vs. 16.3 ± 3.5 dead cells/mm²; P < 0.05; ANOVA). However, the mean effect appeared weaker, but not statistically significantly different from using 5 mg/mL BVC solution (16.3 ± 3.5 vs. 23.0 ± 5.2 dead cells/mm²; not significant). The cell count was reduced by neither 5 nor 10 mg/mL equivalent buffer concentration (41.7 ± 3.3 [5 mg/mL equivalent solvent buffer], 52.9 ± 3.4 [10 mg/mL equivalent solvent buffer] versus 48.3 ± 3.9 [controls] cells/mm²; not significant; ANOVA) (Fig. 4A). Thus, we concluded that the solvent buffer contributed to hTF cell death but alone did not explain the whole effect of 5 mg/mL BVC solution.

Investigation of an Unspecific IgG Effect Using Rituximab. Even though the effect of BVC on hTFs is not related to VEGF binding, it is still conceivable that an IgG (Fc-) cell interaction might promote inhibition of proliferation and cell death. To test this hypothesis we added RTX, an anti-CD20 antibody, to the cell culture. Rituximab is a chimeric (mouse Fab/human Fc) monoclonal IgG1 antibody. However, CD20 is not a specific antigen–antibody interaction with hTFs. We confirmed CD20 negativity of our hTFs by immunostaining and Western blot analysis of hTF- and CD20-positive BJAB cells (Supplementary Fig. S2). Applying RTX to the cell culture, we observed effects comparable to BVC regarding widespread cell death. Morphologically, in the early hours the cells swelled, as with BVC, but later did not completely disintegrate. At 24 hours, their outer shape was grossly retained, while their inner structure appeared dissolved and DAPI nuclear staining was lost (Fig. 5A). The number of cells after 24-hour incubation with RTX under low-serum condition was significantly reduced at a concentration of 5.0 mg/mL, and the number of dead cells
was significantly increased. Both effects were equally strong as with 5 mg/mL BVC (cell counts: 1.2 ± 0.8 [5 mg/mL BVC], 1.5 ± 0.4 [5 mg/mL RTX] versus 20.7 ± 2.4 cells/mm² [control] [P < 0.0001]; ANOVA; dead cells: 12.0 ± 2.6 [5 mg/mL BVC], 11.4 ± 2.1 [5 mg/mL RTX] versus 3.1 ± 1.2 [control] dead cells/mm²; P < 0.01; ANOVA). The number of dead cells increased in a concentration-dependent manner from 1 to 5 mg/mL RTX (Figs. 5B, 5C).
Intracellular Uptake of BVC. Bevacizumab has been shown to be taken up by retinal pigment epithelial cells at much lower concentrations compared to our experiments. We therefore tested whether hTFs also take up BVC. In Western blot analyses of hTF lysate after 6 hours of incubation with different concentrations of BVC, we detected intracellular IgG in a concentration-dependent manner (Fig. 6A). Immunostainings for IgG revealed a perinuclear punctate pattern, which suggested intracellular IgG to be packed in vesicles or to form aggregates (Fig. 6B). However, the uptake of IgG was not detected in all cells. A considerable number of hTFs did not exhibit immunostaining-detectable IgG intracellularly. Moreover, both AFB and RTX were also detected intracellularly (Figs. 6A, 6B). The pattern of distribution and the intracellular amount of IgG protein was comparable to BVC. Thus, we cannot conclude that the intracellular uptake of IgG is per se harmful to hTFs.

hTF Proliferation Was Significantly Reduced by BVC and RTX, But Not AFB

Bevacizumab has been described to inhibit hTF proliferation. Thus, we tested whether VEGF inhibition by AFB could induce a comparable effect and whether the unspecific action of RTX would take place as in the previous experiments. Cell proliferation was measured with BrdU labeling after 24-hour treatment under high-serum conditions (10% FCS). Five milligrams per milliliter BVC and 5 mg/mL RTX significantly reduced cell proliferation compared to untreated controls (percentage of proliferating cells: 29.3% ± 3.6% [5 mg/mL BVC]; P < 0.01; ANOVA and 1.5% ± 1.5% [5 mg/mL RTX]; P < 0.001; ANOVA versus 46.2% ± 2.0% [controls]), whereas 10 mg/mL AFB had no effect (44.9% ± 3.6% [10 mg/mL AFB] versus 46.2% ± 2.0% [controls]; not significant; ANOVA). The difference between BVC and AFB was significant (29.3% ± 3.6% [5 mg/mL BVC] versus 44.9% ± 3.6% [10 mg/mL AFB]; P < 0.01; ANOVA) (Fig. 7), although both substances reduced the level of free VEGF to nearly zero (Fig. 3B). We therefore concluded that the antiproliferative effect of BVC is not based on VEGF inhibition.

DISCUSSION

All previously reported experiments of BVC and hTFs (including ours) were performed with relatively high concent-
trations of the antibody. This increases the chance of observing effects that are not caused by antigen–antibody interaction. Our data supports the hypothesis that this is the case for BVC.

According to our VEGF ELISA measurements 5 mg/mL is already beyond the concentration needed to completely neutralize free VEGF. A concentration of 2.5 mg/mL was equally effective in inhibiting free VEGF but had no effect on our hTF cell culture. Moreover, AFB applied at two different concentrations also reduced free VEGF to almost zero, but neither reduced the number of cells nor increased cell death or inhibited proliferation.

Searching for other, non–VEGF-related effects, we found that the BVC solvent buffer had a significant, but not equally strong, toxic effect on hTFs. Although the number of dead cells was significantly increased, the buffer did not induce the widespread cell loss and cell disintegration observed with the

![Figure 4](https://tvst.arvojournals.org/)

**Figure 4.** Application of antibody-free BVC solvent buffer to quiescent hTFs. Total cell counts (A) and number of dead cells (B) after 24 hours. The addition of BVC solvent buffer in a concentration equivalent to 5 mg/mL BVC significantly increased the number of dead cells but did not reduce the total cell count.

![Figure 5](https://tvst.arvojournals.org/)

**Figure 5.** Incubation of quiescent hTFs with RTX. (A) Phase contrast images at 0, 6, and 24 hours after addition of 5 mg/mL RTX. Within 6 hours the cells swell and the cytoplasm has a highly granular appearance. Within 24 hours the cells lose their inner structure but do not completely disintegrate, as with BVC. Regarding the reduction of the total number of cells (B) and the increase in the number of dead cells (C), 5 mg/mL RTX were equally effective as 5 mg/mL BVC.
BVC solution. Thus, the buffer might have played an adjunctive, but not essential, role. We hypothesize that the low pH (5.9) of the solvent buffer, which was chosen by the manufacturer to prevent aggregation of the antibody protein (personal communication with Roche), was mainly responsible for the increased number of dead cells. Changing the buffer to PBS made BVC less harmful to hTFs (Fig. 4B), but we cannot rule out that this was due to increased aggregation of the protein.

More striking was the application of RTX, after which widespread cell death and inhibition of proliferation occurred. As fibroblasts do not express CD20, a non–antigen–antibody-related mechanism must be the underlying mode of action. We hypothesize that binding of the IgG-Fc part on the hTF surface could trigger the antiproliferative and cell death–inducing effect. Recently, binding of BVC to a neonatal Fc receptor on retinal pigment epithelium cells has been identified as the initial event of intracellular uptake.\(^{19}\) Both BVC and RTX were detected intracellularly in a granular pattern, and the amount of intracellular IgG was positively correlated with the antibody concentration in the culture media. Thus, an overload with IgG might have occurred when applying BVC or RTX at relatively high concentrations. A main counterargument against this hypothesis is, however, that AFB, which had no harmful effect

**Figure 6.** Intracellular uptake of IgG-Fc protein into proliferating hTFs. (A) Western blot analysis of hTF lysate to detect IgG-Fc. GAPDH (37 kDa) served as loading control. Bevacizumab, AFB, or RTX were applied at different concentrations for 6 hours to proliferating hTFs (10% FCS). The proliferative condition was chosen to retain a sufficient number of cells for Western blot analysis as quiescent cells were rapidly destroyed by BVC or RTX. The bands representing the 50- and 25-kD chains of BVC and RTX increased with increasing antibody concentration in the culture media. The AFB protein chains migrated at slightly different sizes, but the band intensity also increased with increasing media concentration of the antibody. (B) Immunostaining for IgG in hTFs exposed to 5 mg/mL BVC, 10 mg/mL AFB, or 5 mg/mL RTX for 6 hours. Intracellular IgG-Fc protein signal was detectable in treated cells but not in nontreated controls.

**Figure 7.** Assessment of hTF proliferation after 24-hour incubation with 5 mg/mL BVC, 10 mg/mL AFB, or 5 mg/mL RTX. 5-Bromo-2-deoxy-uridine incorporation was used as a marker for proliferating cells. The data are presented as the percentage of proliferating cells relative to the total number of cells. Bevacizumab but not AFB significantly inhibited hTF proliferation. Rituximab was even more effective than BVC.
on hTFs, contains an IgG1-Fc domain as well and was taken up intracellularly and distributed in a pattern similar to BVC and RTX. The fact that the latter two are monoclonal antibodies, while AFB is a fusion protein with an IgG1-Fc part, may be a reason for the observed difference. In a previous work on BVC and hTFs, O’Neill et al.10 also tested for an unspecific IgG effect using a humanized monoclonal IgG1 chimeric antibody and a nonhumanized IgG isotype. Their antibody had no significant influence on cell proliferation or survival, though no human antibodies were tested.

Our data challenge the current concept of VEGF inhibition as a strategy for suppressing hTF proliferation. Consequently, the role of VEGF for tenon fibroblasts remains elusive. We hypothesize that the secretion of VEGF from proliferating hTFs is directed toward promoting angiogenesis during wound healing rather than providing a positive feedback loop to enhance hTF proliferation.

In the published in vivo experiments, BVC subconjunctival injection led to significant improvement of bleb morphology and survival.13-25 The antifibrotic effect can, thus, be considered a result of the unspecific IgG-related toxicity we have observed rather than a result of a specific VEGF inhibition. When injecting pure BVC 25 mg/mL solution subconjunctivally, concentrations greater than 5 mg/mL are likely to be maintained at the tenon tissue for a significant period of time. This might have been sufficient to induce fibroblast cell death in a way similar to our in vitro experiments. An additional VEGF-specific mode of action promoting a beneficial outcome could have been suppression of neovascularization and reduction of vascular hyperpermeability (Welsandt G, et al. IOVS 2007;48:ARVO E Abstract 856), both factors contributing to postoperative fibroblast activation and scarring. For these reasons anti-VEGF treatment may still remain a strategy worth pursuing.

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